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• REVIEW •

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Small RNAs in pollen

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In plants, each pollen mother cell undergoes two rounds of cell divisions to form a mature pollen grain, which contains a vegetative cell (VC) and two sperm cells (SC). As a companion cell, the VC carries the SCs to an ovule by germinating a pollen tube. In-depth sequencing analyses of mature pollen showed that microRNAs (miRNAs) and short interfering RNAs (siRNAs) are present in both the VC and SCs. Additionally, epigenetically-regulated transposable elements (TEs) are reactivated in the VC and these TE mRNAs are further processed into 21-nt epigenetically reactivated siRNA (easiRNA) in SCs, which prevent 24-nt siRNA accumulation and sequester miRNA loading. Small RNAs are thought to move from the VC to SCs, where they regulate gene expression and reinforce TE silencing. Here, we summarize current knowledge of the biogenesis and function of miRNAs, siRNAs, and easiRNAs in pollen, emphasizing how these different small RNAs coordinately contribute to sperm cell formation and TE silencing.

vegetative cell, sperm cell, easiRNA, transposable elements

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Plants and animals undergo sexual reproduction to produce the next generation. In angiosperms, gametogenesis derives from microspores in the anthers, unlike in animals, where the haploid germline directly differentiates into gametes. The microspores are formed after the diploid pollen mother cell undergoes meiosis to form four haploid cells. Each microspore first undergoes an asymmetric division, yielding a larger vegetative cell (VC) and a smaller generative cell (GC). The larger VC is maintained at the G1 phase of the cell cycle, but the smaller GC undergoes a second cell cycle and a symmetric mitosis to form two haploid sperm cells (SC). The VC, as a companion cell without any DNA contribution to the zygote or endosperm, delivers two sperms to an ovule by developing a pollen tube. One SC fuses with the egg cell to form the zygote, whereas the other one fuses with the central cell to form endosperm [1].

The asymmetric division of the microspore is an important process during male gametogenesis since an induced symmetric division results in two VCs and no male germline [2]. Very few genes have been identified that affect asymmetric microspore division, possibly because they encode proteins that have important general cellular functions such as cell polarity and cell fate determination. Following asymmetric division of the microspore, several genes have been implicated in the subsequent symmetric cell division that forms twin SCs [3]. Among these, *DUO POLLEN1* (*DUO1*) encodes a male germ cell-specific R2R3 Myb transcription factor that is necessary for twin SC formation [4]. By monitoring the expression of genes associated with germ cell specification and cell differentiation, recent studies [5,6] demonstrated that DUO1 is the key regulator of germline expression of downstream genes in SC formation. In contrast to the myriad *DUO1* targets, how *DUO1* transcription is activated specifically in the GC remains unknown. A recent study [7] showed that an ARID protein acts as a *DUO1* activator to promote SC formation.

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Based on their mode of biogenesis and their functions, small RNAs can be roughly divided into microRNAs (miRNAs) and short interfering RNAs (siRNAs). miRNAs are produced from a single-stranded RNA that base pairs to form a hairpin structure, whereas siRNAs result from the processing of long double-stranded RNAs. miRNA biogenesis depends on Pol II transcription and subsequent processing of stem-loop precursors by Dicer-Like 1 (DCL1), and several other genes have been found to facilitate DCL1 cleavage [8]. 21-nt miRNAs are loaded into Argonaute 1 (AGO1) and act by cleavage of target mRNAs or by translational repression [8].

In contrast, several sub-classes of siRNAs have been identified in plants; some dedicated to the regulation of coding genes (trans-acting siRNAs/tasiRNAs and natural antisense siRNAs/nat-siRNAs), while others are associated with 'non-coding' genomic regions such as hetrochromatin (hetrochromatic siRNA/hcsiRNAs). The biogenesis of tasiRNAs is miRNA-dependent, and an RNA-dependent RNA polymerase 6 (RDR6) then copies the cleaved mRNAs to form dsRNAs, which are generally cut by Dicer-Like 4 (DCL4) to form phased 21-nt tasiRNAs [9]. These tasiRNAs then target specific transcripts in a manner similar to that of miRNAs [9]. Nat-siRNAs arise from loci producing *cis* natural antisense transcript pairs (*cis*-NATs), which in turn regulate the level of expression of the paired transcripts [10]. The biogenesis of hcsiRNAs depends on plant-specific Pol IV (DNA-dependent-RNA polymerase IV), which presumably transcribes a single RNA template from DNA repeated or transposable element (TE) sequences [11]. RDR2 (RNA-dependent RNA polymerase 2) then copies the single-stranded RNA transcript into double-stranded RNA, which will be cleaved into 24-nt siRNA duplexes by DCL3 (Dicer-Like 3) [11]. hcsiRNAs usually silence DNA repeated sequences or TEs by an RdDM pathway [11]: hcsiRNAs are loaded onto AGO4 with the help of scaffold transcripts generated by Pol V (RNA-dependent DNA polymerase V), to guide DNA methylation by recruiting the DNA methyltransferase, DRM2.

So far, small RNAs-mediated epigenetic regulation has been well studied in sporophytic tissues, but few function and mechanism has been known about small RNAs in plant gametes. In this review, based on current knowledge about small RNAs in pollen from several genome-wide studies [12–19], we summarize potential functions of different classes of small RNAs during male gametogenesis, especially focusing on how these different classes of small RNAs coordinately contribute to SC formation and TE silencing.

1 miRNAs in pollen

Although miRNA biogenesis and activity is well characterized in sporophytic tissue, little is known about miRNAs in gametophytic tissue. Seven independent studies [12–18] used microarrays, quantitative reverse transcription-PCR (qRT-PCR), and deep sequencing reported that almost all conserved miRNA families, including miR156, miR159, and miR171, are present in *Arabidopsis* pollen and purified SCs (Table 1). Among these studies, three independent studies [12,16,18] identified more than 20 potentially novel miRNAs in pollen and SCs, and which are enriched variations in the generating region and the sequence length of miRNAs in pollen and SCs. For example, a recent study [18] showed two new classes of miRNAs were encoded by transposons, thus they have named these pollen-specific miRNAs as epigenetically activated miRNAs (eamiRNAs). Consistent with the detection of miRNA in pollen, one of the most important genes, *DCL1*, involved in miRNA biogenesis, is expressed during SC formation [12,13,15] (Table 2). However, although miRNAs have been detected in both the VC and SCs, the biological function of these miRNAs remains unexplored. For example, miR159 is detected in both the GC and SCs [12,13], but the miR159 target, *DUO1* is specifically activated in the GC and stably present in SCs [4–6], which raises a question: how can a miRNA and its target co-exist in the same cell? Together with the facts that many miRNAs are up-regulated in gametophytic cells compared to that in somatic cells [19] and many miRNAs and their targets co-exist in SCs [13,20], these phenomena indicate that miRNAs in plant germlines play an important role in gametogenesis and might have evolved non-canonical mechanism to regulate gene expression.

Different from miRNA biogenesis, which is largely con-

Table 1 Categories of small RNAs in pollen

Category	Size	VN	SN	Target
miRNA family[a)]				
miR156/miR157	21 nt	present	present	SPL
miR158	21 nt	present	present	PPR
miR159/miR319	21 nt	present	present	MTB
miR160	21 nt	present	present	ARF
miR161	21 nt	present	present	PPR
miR162	21 nt	present	present	DCL1
miR163	21 nt	present	present	SAMT
miR164	21 nt	present	present	NAC
miR165/miR166	21 nt	present	present	HD-ZIPIII
miR167	21–22 nt	present	present	ARF
miR168	21 nt	present	present	AGO1
miR169	21 nt	present	present	HAP2
miR170/miR171	21 nt	present	present	SCR
miR172	21 nt	present	present	AP2
miR173	22 nt	present	present	TAS1, TAS2
miR390/miR391	21 nt	present	present	TAS3
eamiRNA	21 nt	present	present	TE
tasiRNA	21 nt	present	present	PPR, ARF
nat-siRNA	21 nt	absent	present	ARI14
hcsiRNA	24 nt	few	few	TE and DNA repeats
easiRNA	21 nt	absent	present	specific TE family

a) indicates only conserved and abundant miRNA family listed in the table.

Table 2 Expression of genes in small RNA pathways during SC formation

Gene	UM	BP	MP
The miRNA pathway			
DCL1[a,b,c]	absent	present	only present in SN
HYL1[a,b,c]	absent	present	both present in VN and SN
SE[a,b]	present	present	absent
TGH[a]	absent	absent	absent
DDL[a]	present	present	absent
SIC[a]	present	present	absent
STA1[a]	present	present	both present in VN and SN
MOS2[a]	absent	present	both present in VN and SN
HEN1[a,b]	present	present	only present in SN
Hasty[a,b]	present	absent	absent
AGO1[a,b]	present	present	only present in VN
AGO5[a,b,c]	present	present	only present in SN
AGO10	present	present	absent
The tasiRNA pathway			
RDR6[a,b]	present	present	present
SGS3[a,b]	present	present	absent
DCL4[a,b]	absent	absent	present
The hcsiRNA pathway			
NRPD1[a,b]	absent	absent	absent
NRPD2[a,b]	present	absent	absent
CLSY[a]	absent	absent	absent
RDR2[a,b]	present	present	only present in SN
DCL3[a,b]	present	present	absent
NRPE1[a,b]	absent	absent	only present in SN
AGO4[a,b]	present	present	absent
AGO6[a,b]	present	present	only present in SN
AGO9[a,b,c]	present	present	both present in VN and SN
KTF1[a]	present	present	absent
DMS3[a]	present	present	only present in VN
RDM1[a]	present	present	absent
DRD1[a,b]	absent	absent	absent
DRM2[a,b,c]	present	present	only present in VN

UM (unicellular microspore), BP (bicellular pollen), and MP (mature pollen) represent three developmental stages, respectively. VN indicates the nucleus of the vegetative cell; SN indicates nuclei of sperm cells. a represents data from microarray and/or RNA-seq analysis; b represents data from RT-PCR analysis; c represents data from transgenic plants of protein fusion.

served in sporophytic and gametophytic tissues, the most important gene in the miRNA effector complex, AGO1, is hardly expressed in SCs [20] (Table 2), suggesting that a potentially unique miRNA silencing pathway is present in SCs. AGO5, which specifically accumulates in SC [16], is a closely related homologue of AGO1 that specifically binds C-initiated small RNAs [21] and could thus be involved in a special miRNA activity in pollen. Moreover, a recent study [22] showed that miRNAs in SCs inhibits expression of targets only at the protein level but not at the mRNA level, which is different from that miRNAs repress targets mainly at the mRNA level in the VC. Taken together, these results clearly indicate that mature miRNAs are present in both the VC and SCs, but the spatial temporal regulation of miRNA activity during SC formation and why the mechanism of miRNA-mediated target repression is distinct between the VC and SCs remain unexplored.

Along with the well-documented siRNA movement between neighboring cells via plasmodesma [23], several lines of evidence indicate that miRNAs are also on the move [24]. It was recently discovered [24] that miRNAs produced in root cells can move into adjacent cells, possibly via plasmodesma, and finally enter aerial parts to regulate target expression in the phloem. Therefore, there is a precedent to consider that miRNAs in pollen could move between the VC and SCs. Indeed, by expressing artificial miRNAs against green fluorescent protein (GFP) under the control of the VC-specific promoter *LAT52*, which is also somewhat expressed at earlier stages, it was possible to target *GFP* expression in the SCs [15]. However, a recent study [22] argued that miRNA in SCs are only inherited from the microspore due to cell division, instead of moving from VC to SCs, because the authors found no obvious reduction in the GFP signal in sperm cells by introducing artificial miRNAs against *GFP* under the control of the later stage VC-specific promoter *VCK1*, which was only activated after the first cell division. Future detailed studies will be necessary to conclude whether miRNAs move or not, and even though miRNAs move in pollen, the mechanism of movement between the VC and SCs might be different from that in those sporophytic cells, because SCs are located in the cytoplasm of the VC, and SCs lack a cell wall.

2 tasiRNAs and nat-siRNAs in pollen

Small RNA deep sequencing analysis showed that tasiRNAs, generated from all four TAS transcripts, can be detected throughout male gametophyte development [13] (Table 1). Moreover, both the accumulation and varieties of tasiRNAs were significantly increased as pollen development proceeded [13]. Both RNA-seq [20] and qPCR analysis [15] confirmed RDR6 and DCL4 are expressed throughout pollen development (Table 2), indicating the presence of the tasiRNA pathway plays a role during SC formation. Future work focusing on the function of these tasiRNAs during pollen development will further elucidate how tasiRNAs are coordinated with other small RNAs to promote SC formation.

RNA-seq analysis showed more than 30 pairs of transcripts exhibit inverse patterns between the VC and SCs (Qin P and McCormick S, personal communication), suggesting the potential for nat-siRNAs from these paired genes in mature pollen. A recent study from the McCormick group demonstrated that the misregulation of a specific natsiRNA pair in *Arabidopsis* SCs resulted in impaired double fertilization [25]. The authors found the overlapping transcripts from the pair of *kokopelli* (*KPL*) and *ARIADNE14* (*ARI14*) exhibited inverse expression patterns between the VC and SCs, and that this pair indeed gave rise to the production of natsiRNAs that down-regulate *ARI14* in SCs [25]. *KPL* encodes a unknown protein, while ARI14 is a putative inactive E3 ligase that was hypothesized to com-

pete with two other homologs with functional E3 ligase activity for substrates [25]. Future work to investigate functions of other pairs in pollen will help us to understand more broadly the significance of natsiRNAs during SC formation.

3 hcsiRNAs in pollen

TEs are regarded as "selfish DNA", and TEs occupy a huge proportion of eukaryotic genomes, thus preventing the random jumping of these invasive TEs is thought to be essential for genome stability. Specifically, keeping TEs silenced during fertilization is important for the viability and development of offspring, because if the egg cell or SCs carried unexpected insertion or deletions in the genome, it could lead to the failure of embryo development or affect postembryonic development. In animals, piRNAs play a major role in TE silencing in germlines [26]. However, no piRNA is found in plants, and 24-nt small RNAs occupy more that 70% of total small RNAs, and among these, hcsiRNAs are the most abundant class that are well-known players to silence TEs [11]. However, TEs are reactivated in the VC but silenced in SCs of pollen [15], which accompanied with accumulation of many 21-nt siRNAs but few 24-nt siRNAs originated from TEs detected in SCs but not in the VC [15] (Table 1). Therefore, whether and how these 21-nt hcsiRNAs instead of 24-nt hcsiRNAs are responsible for distinct patterns of TE expression between the VC and SCs remain unknown.

Besides the hcsiRNA-mediated RdDM pathway for TE silencing, DDM1 (decreased in DNA methylation 1) is a chromatin remodeling protein that is responsible for TE silencing in plants [27], because the loss of *DDM1* results in globally reactivation of TEs and the accumulation of 21-nt siRNAs but not of 24-nt siRNAs [15,18]. Therefore, the transient loss of *DDM1* expression in the VC and the presence of *DDM1* expression in SCs were thought to be why TEs are reactivated in the VC but silenced in SCs. However, 21-nt siRNAs are not generated equally from all reactivated TEs but only from several TE families [15,18], indicating other mechanisms besides siRNA-directed epigenetic regulation are involved in TE reactivation in the VC.

In plants, hcsiRNA-mediated TE silencing is usually accompanied with CHH DNA methylation, i.e., RdDM. However, the overall level of CHH methylation is maintained in the VC but lost in SCs, and that of CG and CHG methylation are not different in the VC and SCs [17], which does not explain the reactivation of TEs in the VC and silencing of TEs in SCs. No correlation between DNA methylation and TE activity indicates that the maintenance of DNA methylation (CG, CHG, and CHH) does not interfere with TE reactivation in the VC, and the loss of CHH methylation also does not release TE silencing in SCs. These observations suggest that 21-nt siRNAs, unlike hcsiRNAs, have evolved a distinct mechanism of TE silencing in pollen. It is possible that histone modifications instead of DNA methylation might be mainly associated with TE activity in pollen. Moreover, many important components such as Pol V and AGO4 in the RdDM pathway are gradually down- regulated in mature pollen [15,20] (Table 2), suggesting that a unique silencing pathway for TE activity has been established during SC formation.

4 Epigenetically reactivated siRNAs (easiRNAs) in pollen

Why do 21-nt siRNAs strongly accumulate in pollen, but only a few 24-nt siRNAs? A recent study [18] confirmed that these 21-nt siRNAs are generated from reactivated TEs in pollen. After being transcribed by Pol II, TE mRNAs are targeted by canonical miRNAs to trigger production of 21-nt siRNAs by RDR6 and DCL4 [28,29]. These 21-nt TE-derived siRNAs are named as easiRNAs [18]. Consistent with the notion that easiRNA biogenesis is similar to that for tasiRNA, the accumulation of easiRNAs in *ddm1* mutants was abolished by *dcl1* or *rdr6* mutations [18]. Based on their similarities of biogenesis, easiRNAs are also very similar to 21-nt phased siRNAs (phasiRNA) [30,31]. Furthermore, in rice over 1,000 phasiRNAs were preferentially produced in male reproductive organs [30]. Taken together, exploring the biological significance of easiRNAs or phasiRNAs will help us to further understand how *de novo* invading TEs are perceived by their hosts and then how the host maintains TE silencing in subsequent generations.

Because easiRNAs are generated from reactivated TEs, easiRNAs are only detected at specific developmental stages, such as in the VC [15], or are induced by specific stress conditions [32]. The original purpose of easiRNAs during evolution might have been in response to stress stimuli or specific developmental stages, which increases phenotype diversity or even facilitates the evolution of new species [33]. Another purpose of easiRNA accumulation might be to regulate expression of coding genes in *trans* [34], which is similar to the action of tasiRNAs. Genome-wide profiling analysis identified 27 candidate genic mRNAs that do not contain a TE fragment but are regulated through partial complementarity by accumulation of easiRNAs [28]. For example, one easiRNA specifically targets and inhibits the formation of a host protein that acts to repress TE activity, suggesting that in an evolutionary sense, TEs harbor and potentially select short sequences to act as suppressors of host TE repression [28].

The reactivation of TEs in the VC and the existence of easiRNAs in SCs in pollen also suggest that they are relevant to each other. The prevailing model is that easiRNAs are generated in the VC, then transferred into SCs [15], but the mechanism of transfer remains elusive. Given that SCs are produced from the precursor GC by cell division,

easiRNAs could be first generated in the GC and then experience further amplification in SCs, which is similar to the biogenesis of phasiRNA [30,31]. Our recent studies [7,35] showed that both Cajal bodies, the siRNA processing center, and ARID1, an epigenetic component associated with histone deacetylases, are specifically located in the GC but not SCs, suggesting that the distinct epigenetic environment between the VC and SCs might have been established at the bicellular stage. Therefore, further detailed comparative analysis of easiRNA accumulation in the VC, the GC and SCs will shed light on when and how TEs are preferentially reactivated in the VC, and how active TEs in the VC are recognized by easiRNA-processing machinery, such as Pol II, RDR6, and DCL4.

5 Antagonistic effects of different classes of small RNAs in pollen

That transcribed TE mRNAs contain many canonical miRNA binding sites and that miRNA-mediated TE mRNA cleavage leads to easiRNAs production suggests that different classes of small RNAs established an unexpected link during SC formation (Figure 1). DCL1 and RDR6 are necessary for tasiRNA biogenesis. Compared to that in *ddm1* mutants, global easiRNA levels were indeed lower in *ddm1 dcl1* double mutants and, as expected, were nearly abolished in *ddm1 rdr6* double mutants [18]. Further mapping of potential 21-nt easiRNAs showed that approximately 1,000 TE mRNAs possibly undergo miRNA-mediated cleavage and simultaneously generate easiRNAs in inflorescences of the *ddm1* mutant [18].

How do easiRNAs function *in vivo*? A previous study showed that easiRNAs are loaded onto AGO1 and thereby prevent miRNA loading [28], which provides direct evidence that easiRNAs are antagonistic with miRNAs. Moreover, analysis of total 21-nt small RNAs accumulation in *ddm1* mutants showed that although the total reads of 21-nt small RNAs remain similar, 21-nt miRNAs were greatly reduced while accumulation of 21-nt easiRNAs increased [28]. Further work focusing on what specific feature of TE mRNAs attracts the easiRNA processing machinery will clarify how the biogenesis and loading of miRNAs are inhibited in the presence of easiRNAs.

The finding of miRNA-dependent easiRNAs in pollen provides another interesting hypothesis about the origin of miRNAs. Many prevalent easiRNA-generating miRNAs are highly conserved across plant species, and these miRNAs were possibly generated during the initial invasion of genomes by TEs, which might have been accompanied by inversion-duplication events. Consistent with these co-occurrence events, both epigenetically reactivated miRNAs (eamiRNAs) and easiRNAs are detected in the *ddm1* mutant [18]. Therefore, the initial purpose of miRNA production raises the possibility that, just like siRNAs, miRNAs might have possibly evolved first to defend against further TE invasion rather than as regulatory elements. Subsequently, with the integration of TE sequences into their host genome and optimizing genome stability, these reconstituted DNA sequences could be recognized and transcribed into 24-nt hcsiRNAs, which evolved specifically to maintain TE silencing. In contrast, miRNAs might have evolved a new function to regulate gene expression, instead of solely stably repressing TEs by hcsiRNAs, with the exception of specific developmental stages such as in pollen.

In addition to the antagonistic interaction between easiRNA and miRNA, in-depth analysis of small RNA sequences showed that the accumulation of easiRNAs obviously led to the reduced accumulation of 24-nt siRNAs [18,28]. The antagonistic role of 24-nt siRNA biogenesis by easiRNAs was more obvious in *ddm1* mutants [18,28], since easiRNAs accumulated not only in pollen but also in spo-

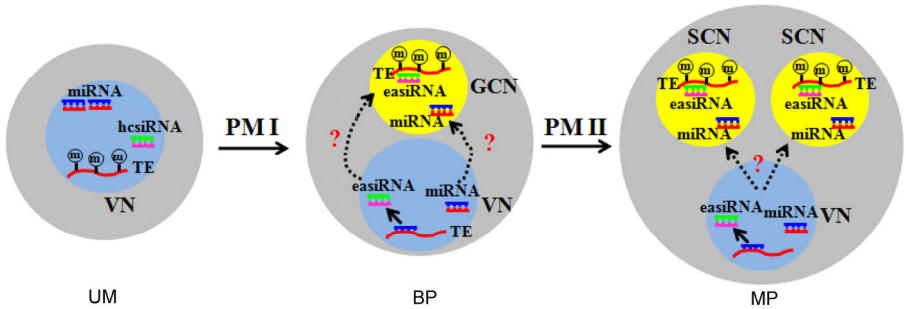

Figure 1 (color online) Small RNAs during sperm cell formation in plants. miRNAs are present in both the VN (vegetative nuclei), GCN (generative cell nuclei), and SCN (sperm cell nuclei) during pollen development. In contrast, 24-nt siRNAs are barely detected in mature pollen and SCs, indicating that 24-nt siRNAs accumulation is inhibited in male gametes. However, TE-derived 21-nt siRNAs (easiRNAs) are most likely produced from activated TEs in the VC of bicellular pollen (BP). easiRNAs, together with miRNAs, move to GCN by an unknown mechanism. After the second mitosis (PM II), two SCs and the VC are inherited directly from the pre-established pattern of small RNAs in the BP stage. Coinciding with the switch of small RNAs accumulation during SC formation, TEs are silenced in the unicellular microspore (UM) stage, due to the presence of 24-nt siRNAs. When pollen development proceeds to the BP stage, TEs are reactivated, possibly by responding to an unknown cue, and activated TE loci are processed into easiRNAs by the well-known Pol II-miRNA-RDR6 pathway. The precursor TE mRNAs or their product easiRNAs move away from the VC, then easiRNAs assume the role of 24-nt siRNAs to silence TEs in GCN and SCs.

rophytic tissues [18,28]. How easiRNAs are antagonistic with 24-siRNAs will be a major focus in the future. Because most TE families in *Arabidopsis* do not generate easiRNAs, how these TE family-derived easiRNAs can globally inhibit the accumulation of 24-siRNAs is unclear. A possible avenue towards an answer is to examine the substrate specificity of RDR2 and RDR6. A prevailing model is that RDR2 facilitates the recognition or transcription of inactive TE loci by Pol IV [11], whereas RDR6 and Pol II process active TE mRNAs into easiRNAs [18]. Whether RDR6/easiRNAs affects the interaction between RDR2 and Pol IV or the transcription activity of Pol IV/RDR2 remains unknown.

6 Perspective

With the identification of new classes of small RNAs in the plant germline, understanding the biological significance of these different small RNAs in gametes becomes increasingly important. Why 21-nt and not 24-nt siRNAs are accumulated in SCs? And are these small RNAs with specific sizes associated with unequal epigenetic modifications between the VC and SCs? In female gametes, there are 24-nt hcsiRNAs in the egg cell but not in the companion cell, so what is the trigger for such a big difference between male and female gametes? And how are 21-nt siRNAs from SCs coordinated with 24-nt hcsiRNAs from the egg cell during double fertilization? Therefore, although no piRNAs are present, plants have evolved a complicated mechanism to maintain TE silencing during reproductive stages through integrating miRNAs and siRNAs.

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